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Wound Healing Potential of Diterpenoids from the Hexane Fraction of *Kaempferia galanga* Rhizomes

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ABSTRACT

Kaempferia galanga, a member of the *Zingiberaceae* family, is widely cultivated throughout Southeast Asia and traditionally used for the treatment of inflammation, pain, and dyspepsia. This study aimed to isolate compounds responsible for anti-inflammation and wound treatment from *K. galanga*. Bioassay-guided isolation of ethanol extract was used in this study. The hexane fraction from ethanol extract of *K. galanga* demonstrated significant activity by inhibiting nitric oxide released from LPS-stimulated RAW264.7 cells. It was then isolated to obtain the active compounds. Ten diterpenes (KG1-10) isolated from this fraction were investigated to determine their wound healing properties including anti-inflammatory effect using RAW264.7 cells, amelioration of human dermal fibroblasts (HDF) proliferation and migration, effect on collagen production and oxidative stress in H_2O_2-induced HDF cells. It was found that 6β-acetoxysandaracopimaradiene-1α, 9α-diol (KG6) displayed the most capable anti-inflammatory effect, with an IC_{50} value of 18.7 µM. This compound also showed good cell proliferation and migration on day 3 at 116.5% and 88.5%, respectively. Furthermore, 6β,14α-dihydroxyisopimara-8(9),15-diene (KG8) meaningfully increased the viable cell (119.7%) and extended migration of HDF cells (88.4%) which was preferable effect than that of allantoin (102.4, 75.5%). 6β-Acetoxysandaracopimaradiene-9α-ol (KG2) significantly increased the collagen content (38.7 µg/ml) in HDF cells. In addition, 1α-hydroxy-14α-methoxyisopimara-8(9),15-diene (KG7) exhibited the highest cytoprotective activity by increasing the viability of H_2O_2-induced oxidative damage in HDF cells to 92.1%. The hexane fraction was analyzed using gas chromatography to identify the presence of active wound healing compounds, KG1-10. This fraction showed significant potential as a pharmaceutical ingredient in wound treatment products due to its strong wound healing properties.

Keywords: *Kaempferia galanga*, Wound healing, Anti-inflammation, Antioxidant, Diterpenes.

Introduction

Skin is a barrier of body that protects internal organs from injury or any pathological conditions. The damage of epithelial or subcutaneous tissue must be rapidly and efficiently mended to maintain tissue homeostasis. The healing of cutaneous wound is a cellular dynamic and complex process which including homeostasis and inflammation, re-epithelialization and formation of granulation tissue, and extracellular matrix reconstruction.[1,2]This well-organized process requires the orchestration of many different cell types to initiate the healing process such as macrophages, fibroblasts, keratinocytes and capillaries.[2,3] Fibroblast is an abundant cell in skin tissue and one of the key cells of the wound healing process.

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It plays an important role, including rupturing of fibrin clots, extracellular matrix components synthesis and generation of collagen to support the tissue homeostasis.[2,3] Collagen and granulation tissue formation play a pivotal role in wound contractions that reduce the open wound and mature the granulation tissue.[3] In addition, epithelial cells and angiogenic cytokines establish microvascular network to supply the oxygen and nutrients in this phase.[4] And lastly, the remodeling phase is reformation and arrangement of collagen fiber components for escalation the tensile strength. Though the wound healing process is a natural response after injury, it can be impaired by several factors including microbial infection, excessive inflammation and reactive oxygen species, resulting in a chronic wound that fail to promote usual stepwise healing progression.[5]

Medicinal plants and new bioactive compounds are needed to expedite the treatment of various wound types. A large number of plants used in traditional wound healing medicine in many countries have attracted research interest for their constituents and mechanisms of action. Increasingly, the search for new plant-based therapeutic agents for wound healing is shifting towards extracts or compounds with multi-target potential, having impact for example on inflammation, proliferation and tissue remodeling phases.[5]*Kaempferia galanga* L. or aromatic ginger belongs to the *Zingiberaceae* family. The plant has much gained attention owing to its medicinal properties, including anti-inflammatory, anti-nociceptive, anti-oxidant, anticancer, antimicrobial, anti-diabetic, insecticidal and repellent activities.[6]*K. galanga* rhizomes

have been widely used in Thai remedies for treating skin infections, dyspepsia, stimulating menstrual cycle, as a cardiotonic, and central nervous system stimulant.[7] Moreover, it has been used to treat headaches, fever, pain, skin diseases, inflammation, wounds, and mouth sores and blisters.[6]*K. galanga* contains essential oils, phenolic compounds, flavonoids, diarylheptanoids, terpenoids, and other compounds. These compounds have also been reported their pharmacological effects such as terpenoids exhibit a good effect on pain management, inflammation, and wound care.[6] The biological potential of diterpenes including anticancer, anti-oxidant, anti-allergic, antimicrobial, wound healing, and anti-inflammatory effects, has been demonstrated.[8-11] However, reports on the wound healing properties of diterpenes isolated from this plant are quite limited. The aim of this study was to evaluate the wound healing potential of diterpenes isolated from *K. galanga* rhizomes. Specifically, we sought to determine how these compounds might promote wound repair and recovery. The relevance of the research methods lies in their ability to demonstrate the potential of test samples across various wound-healing phases, as the assays used are designed to closely mimic actual wound healing conditions. Consequently, the impact of the diterpenoids on various vital aspects of wound healing, including anti-inflammatory activity, cellular proliferation, collagen production, and antioxidant activity against hydrogen peroxide (H_2O_2) induced oxidative stress, were investigated.

Materials and Methods

Reagents and Chemicals

All reagents and chemicals were of analytical grade. Various essential culture media and reagents such as RPMI 1640 medium (RPMI), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, 0.25% trypsin-EDTA, Trypan blue, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and phosphate-buffered saline (PBS) were obtained from Gibco® (Life Technologies, Paisley, Scotland). Reagents, including lipopolysaccharide (LPS), Griess reagent, indomethacin, L-nitroarginine (L-NA), caffeic acid phenethyl ester (CAPE), allantoin, dimethyl sulfoxide (DMSO), and hydrogen peroxide (H_2O_2) were sourced from Sigma-Aldrich (Missouri, USA).

Plant material and isolated compounds from K. galanga

K. galanga rhizomes were bought in April 2019 from Prachinburi province (14˚ 3′ 0″ N, 101˚ 22′ 48″ E, Thailand). The sample was identified by The Royal Society of Thailand, Bangkok, Thailand as *Kaempferia galanga* (Herbarium No. SKP 201110701). Thereafter, 3 kg dry weight of *K. galanga* rhizomes was ground and extracted by reflux with 95% ethanol to yield crude ethanol extract. The extract was suspended in 95% methanol and partitioned with hexane to give the hexane fraction. After that, the marc was evaporated and dissolved in water and was subsequently partitioned to give chloroform, ethyl acetate and water fractions, respectively. The obtained fractions were kept in airtight containers at 4°C until use. Bioassay-guided isolation was done. The hexane fraction displayed good wound healing activity and it was subsequently chromatographed. The constituents of this fraction were isolated and identified as previously described by Tung charoen and colleagues.[8]

Cell cultures and sample preparations

At 37°C, 5% CO_2 in humidified atmosphere incubator, RAW264.7 cells were cultured in RPMI and Human dermal fibroblast (HDF) cells were cultured in DMEM. Both media were supplemented with 10% FBS and 1% penicillin-streptomycin. Sample stock solutions were dissolved in DMSO and diluted with specific cell media (DMEM or RPMI) to a final concentration of 0.78-100 µg/ml for crude extract and 0.78-100 µM for pure compounds. In the preliminary test, at concentration of 0.5% DMSO diluted in media alone did not show cytotoxic effect to the cells throughout this study. Thus, the final concentration of DMSO in each sample was less than 0.5% before use.

Anti-inflammation assay using LPS-induced RAW264.7 cells

LPS-induced RAW264.7 murine macrophage cells to produce nitric oxide (NO). The excessive production of this molecule leads to inflammation. The suppression of NO production was assayed as previously depicted by Longsri and colleagues.[12] RAW264.7 cells (1×10^5 cells/well) in RPMI were treated with 0.1 µg/ml of LPS concurrently with the test samples and were then kept for 24 h. NO produced by RAW264.7 cells was examined using Griess reaction method. The absorbance was recorded at 570 nm by SPECTROstar^{Nano} microplate reader (BMG LABTECH, Offenburg, Germany). Indomethacin, L-NA and CAPE were used as positive controls. The percentage of inhibition was calculated by equation 1. The IC_{50} values were determined graphically.

$$\% \text{ Inhibition} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})}{\text{Absorbance}_{\text{control}}} \times 100 \quad [1]$$

After 24 h of incubation with various concentrations of test samples, the viability of RAW264.7 cells was determined using MTT assay.[13] MTT solution (5 mg/ml, 10 µl) was added in each well and incubated at 37°C for 2 h. Thereafter, formazan crystal formed in the viability cells were dissolved by adding DMSO to the wells and the absorbance of the solutions were recorded at 570 nm by microplate reader. The cytotoxic sample was deduced from viability of RAW264.7 cells that was less than 80% of vehicle-treated group.

HDF proliferation assay

Seeding of HDF cellsin DMEM containing 10% FBS at 1×10^4 cells/well into 96-well plate were incubated for 24 h. Thereafter, the cells were treated with different concentrations (0.78-100 µg/ml and 0.78-100 µM) of test samples and were then incubated for 48 h. The viability of HDF cells was determined using MTT assay as described above. The percentage of cell proliferation was calculated following equation 2. Allantoin and *Aloe vera* gel were used as positive controls.

$$\% \text{ Proliferation} = \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100 \quad [2]$$

HDF migration using scratch assay

Study of cell migration using *in vitro* scratch wound assay was performed.[13] Briefly, HDF in DMEM containing 2% FBS were seeded into 24 well plates (5×10^4 cells/well) and incubated at 37°C with 5% $CO_2$to confluence monolayer. HDF cells were horizontally scratched with 1000 µl sterile pipette tip. Any cellular debris was rinsed with PBS and removed from wells. The cells then were treated with test sample. Migration of HDF cells were photographed at day 0 (d_0) to day 3 (d_x) using Eclipse TS100 inverted microscope. Broaden of cells layer toward the lacerated area were analyzed using NIS-ElementsD4 software. Decreasing of the open area percentage indicates the migration of cells. Allantoin and *Aloe vera* gel were used as positive controls. The percentage of cell migration was calculated following equation 3.

$$\% \text{ Migration rate} = \frac{(\text{Gap distance}_{d0} - \text{Gap distance}_{dx})}{\text{Gap distance}_{d0}} \times 100 \quad [3]$$

Soluble collagen production using HDF cells

HDF cells in DMEM containing 2% FBS were seeded into 96-well plate (1×10^4 cells/well). After 24 h, the test samples were added into the well. The cells were maintained in this condition for 48 h at 37°C in 5% CO_2. Sircols Collagen Assay Kit (Bi-color Life Science) was used according to the manufacturer's instructions to detect the production of Type-I collagen. This soluble collagen was dissolved with alkali reagent and was measured at540 nm. The amount of collagen was calculated based on a standard curve of the soluble collagen.

Hydrogen peroxide (H_2O_2)-induced oxidative stress using MTT assay

HDF cells in DMEM (10% FBS) were seeded into 96-well plates (5×10^3 cells/well). After 24 h, the confluence cells were treated with various concentrations (3.12-50 µM) of the compounds. After kept in incubator for 1 h, the cells were challenged with 0.9 mM of H_2O_2 for

another 24 h. Finally, the cell viability was determined using the MTT assay. Allantoin was used as a positive control.

Gas chromatographic (GC) analysis of the hexane fraction from K. galanga rhizomes

The GC analysis was performed using Agilent 7890A GC with split/splitless injector equipped with a HP-5 capillary column (30 m × 0.32 mm × 0.25 µm, Agilent Technologies Inc. USA). Helium, a carrier gas was set at constant flow of 1.0 ml/min. Twenty-five minutes of oven temperature program was initially 60ºC held for 2 min and increased to 250ºC at 15ºC min^{-1}, and then to 305ºC at 5ºC min^{-1}.[14] The injection of each sample (1 µl) was done in the splitless mode. Standards (KG1-10) and hexane fraction at concentration of 1 mg/ml were dissolved in methanol. The samples were filtered through 0.45 µm nylon filters prior to GC-analysis. The fingerprint chromatogram of hexane fraction was performed by comparison with the retention time of standard compounds.

Statistical analysis

All data values were expressed as mean ± S.E.M. of three determinations. The data analysis was achieved by one-way analysis of variance (ANOVA), followed by Dunnett's test using SPSS version 26. The *p* values <0.05 were considered to be significant.

Results and Discussion

Isolation of compounds from K. galanga

Bioactive compounds in *K. galanga* are phenolic compounds, flavonoids, diarylheptanoids, essential oils, and terpenoids. The most abundant constituents found in the *K. galanga* are terpenoids especially diterpenoids with many publications related to these compounds.[7,8,10,11] Moreover, previous phytochemical studies on *K. galanga* rhizomes have demonstrated the presence of various isopimarane diterpenoids in addition to phenolic compounds from this plant.[8,10,11] These compounds have been reported for their anti-inflammatory, antioxidant and antimicrobial activities.[6]Accordingly, *K. galanga* is regarded to attention in aimed at determining the active compounds that are responsible for its wound healing properties. In the present study, yield of *K. galangal* ethanol extract was 9.73% w/w (282.2 g). The hexane, chloroform, ethyl acetate, and water fraction of this extract were yielded 26.2, 45.7, 0.57 and 27.6% w/w, respectively. The ethanol extract and fractions from *K. galanga* showed influential inhibition on NO production (Table 1). Based on bioassay-guided isolation, hexane fraction showed the best activity against NO release, it was selected for compound isolation. The hexane fraction was separated using chromatographic techniques to obtain ten isopimarane diterpenes (KG1-10). The structures of KG1-10 were elucidated by comparing their ^{1}H and ^{13}C NMR spectral data with those of pure compounds as previously described by Tungcharoen and colleagues.[8] The phytochemical characterization of these compounds revealed the isopimarane diterpene type including (-)-sandaracopimaradiene (KG1), 6β-acetoxysandaracopimaradiene-9α-ol (KG2, kaempulchraol K), sandaracopimaradiene-7α, 9α-diol (KG3, boesenberol I), sandaracopimaradiene-1α, 9α-diol (KG4), 6*β*-acetoxysandaracopimaradiene-9α-ol-1-one (KG5), 6*β*-acetoxysandaracopimaradiene-1α, 9α-diol (KG6), 1α-hydroxy-14α-methoxyisopimara-8(9),15-diene (KG7), 6*β*,14*α*-dihydroxyisopimara-8(9),15-diene (KG8, kaempulchraol C), 1*α*,14*α*-dihydroxyisopimara-8(9),15-diene (KG9), and 6*β*,14*β*-dihydroxyisopimara-8(9),15-diene (KG10, kaempulchraol D)(Figure 1).As earlier mentioned, in the search for new therapeutic agents from plant-based remedies for wound healing, the extracts and compounds that impact at least two different phases of wound healing process are preferable.[5] Therefore, the ethanol extract and its fractions from *K. galanga* rhizomes together with isolated compounds were evaluated for their efficacy across the three phases of the wound healing process.

Effect of fractions and compounds on NO production

Inhibition on NO release from LPS-stimulated RAW264.7 cells was done for investigate an anti-inflammatory property of *K. galanga*. The inflammation is induced by the releasing of inflammatory mediators such as eicosanoids, prostaglandins and leukotrienes and reactive oxygen species.[5] LPS from Gram-negative bacteria is widely used to induce inflammation by binding with toll-like receptors 4 to form complex and then stimulate nuclear factor-kappa B (NF-κB). The activated NF-κB plays an important role in pro-inflammatory gene transcription and expression, including cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α) and interleukin (IL-1, IL-6). An overwhelming activation macrophage by LPS yields an excessive variety of inflammatory cytokines and mediators such as IL-1β, IL-6, TNF-α, PGE2, and NO. A high amount of NO is a strong oxidant that can cause damage to normal cells and surrounding tissues, and subsequently escalating the inflammation.[15] Thus, the pharmacological need is the agent that able to inhibit extreme synthesis and function of inflammatory mediators and also reduce tissue damage from free radicals. The anti-inflammatory potential of the test samples was compared with the standard positive controls L-NA (iNOS inhibitor), indomethacin (non-selective cyclooxygenase, COX-1 and COX-2 inhibitor), and CAPE (NF-κB inhibitor). The results showed that the ethanol extract of *K. galangal* showed good NO inhibitory effect in RAW264.7 macrophage cells (IC_{50} = 19.1 µg/mL). The hexane fraction showed the most potent inhibitory effect followed by chloroform-, ethyl acetate- and water fractions with IC_{50} values of 10.1, 13.4, 37.3 and >100 µg/ml, respectively (Table 1). The hexane fraction (IC_{50} = 10.1 µg/ml) exhibited higher effect than L-NA (IC_{50} = 16.6 µg/mL) and indomethacin (IC_{50} = 14.4 µg/ml) but lower than CAPE (IC_{50} = 1.0 µg/mL). Cytotoxic effects were observed from the ethanol extract, hexane and chloroform fractions with LC_{50} values of 33.8 µg/ml, 21.5 µg/mL and 29.1 µg/ml, respectively. The ethyl acetate fraction demonstrated less cytotoxic effect at concentration of 100 µg/ml while cytotoxic effect of the water fraction was not revealed. Ten active compounds of isopimarane diterpenes (KG1-10) isolated from hexane fraction were evaluated for their anti-inflammatory effects. KG6 exhibited the most potent NO inhibition with an IC_{50} value of 18.7 µM, followed by KG8, KG5 and KG10 with IC_{50} values of 19.2, 29.1, and 33.4 µM, respectively. KG6 and KG5 showed significantly influential effect than the positive controls, L-NA (IC_{50} = 71.2 µM) and indomethacin (IC_{50} = 40.3 µM). However, the cytotoxic effects of KG6, KG8 and KG5 were presented at concentration of 100 µM, whereas KG10 displayed an LC_{50} of 56.2 µM (Table 1). KG5-6, KG8 and KG10 exhibited the most potent anti-NO production with better effect than L-NA and indomethacin and yet less than CAPE. In a previous study, it was reported that 6β-acetoxysandaracopimaradiene-9α-ol-1-one and 6β-acetoxysandaracopimaradiene-1α,9α-diol isolated from *K. galanga*significantly down-regulated COX-2 and iNOS mRNA expressions.[8] It is inferred that anti-inflammatory effect of these compounds may exert via the inhibition of prostaglandin and NO synthesis. The mechanism of actions are mediated through the inhibition of converting enzymes COX-2 which converts arachidonic acid to prostaglandin, and iNOS which converts L-aginine to NO and L-citruline, respectively. Therefore, potent anti-inflammatory activity of KG6 and KG8 indicated the suitable for use to reduce inflammation in the first phase of wound healing.

Effect of fractions and compounds on proliferation using MTT assay

Once inflammation is in control, the cell proliferation and tissue regeneration ensue. Fibroblast is an essential cell for wound closure and help to restore the physical function of skin. It plays an important role from the early phase of inflammation to the final of epithelialization. Contribution of fibroblasts involve cell proliferation and migration, and secretion of cytokines, growth factors, collagens and others extracellular matrix components.[16]In consequence, the agent that able to enhance fibroblasts activity is need. Prior, samples were assessed their cytotoxicity on HDF cells using MTT assay for discard any interference on proliferation and migration. The ethanol extract and its fraction at higher concentrations than 25 µg/ml were significantly decreased HDF cell viability, whereas, at lower concentrations (0.78-1.56 µg/ml) the viable cell was increased (Table 2). After 48 h of culturing with 0.78 and 1.56 µg/ml of ethyl acetate fraction, substantial cell proliferation was observed when compared with the untreated

control group. In the presence of ethanol extract, hexane-, chloroform- and water fractions, HDF cell viability was comparable to the control group. The cytotoxic effects were not observed in the treatment group of various concentrations (0.78-25 µM) of KG1-KG10. Among these, KG8 at concentrations of 1.56-25 µM and KG6 at concentration of 6.25-12.5 µM significantly induced HDF cell proliferation up to 107.2-119.7% compared with untreated, allantoin and *Aloe vera* gel groups (Table 3). In addition, these effects increased in a dose-dependent manner. The productive effects were also observed at lower concentrations (1.56 µM) of KG5 and 7. The proliferations of HDF cells in other treated groups were similar to the control groups. Therefore, KG6 and KG8 promoted the wound healing. The results showed that the hexane fraction of *K. galanga* exhibited good wound healing properties due to its constituents. KG6 and KG8 that exhibited the potent anti-inflammatory activity together with KG7 and KG10 promoted human dermal fibroblast proliferation.

Table 1: Anti-nitric oxide production and lethal concentration on RAW264.7 cells

Samples	Anti-nitric oxide production (IC_{50})		Lethal concentration 50 (LC_{50})	
	µg/ml	µM	µg/ml	µM
Ethanol extract	19.1±0.8	-	33.8±0.3	-
Hexane fraction	10.1±0.3[b]	-	21.5±0.2	-
Chloroform fraction	13.4±0.5	-	29.1±0.7	-
Ethyl acetate fraction	37.3±3.1	-	>100*	-
Water fraction	>100	-	>100	-
KG1	-	46.4±1.6[b]	-	>100*
KG2	-	81.8±8.3	-	>100
KG3	-	55.2±3.1[b]	-	>50*
KG4	-	57.0±1.8[b]	-	>100
KG5	-	29.1±3.2[b]	-	>100*
KG6	-	18.7±1.4[a, b]	-	>100*
KG7	-	60.6±4.2	-	>100*
KG8	-	19.2±0.5[a, b]	-	>100*
KG9	-	39.5±0.2[b]	-	>100*
KG10	-	33.4±0.8[b]	-	56.2±1.2
Indomethacin	14.4±1.2	40.3±3.4[b]	>100*	>100
L-NA	16.6±0.7	71.2±3.1	>100	>100
CAPE	1.0±0.1	3.4±0.2	16.2±0.7	57.5±0.8

Each value represents mean±S.E.M. of three determinations.
*Cytotoxic effect was observed.
Statistical significant difference between IC_{50} of positive control and sample at $p< 0.05$. Dunnett t-tests treated one group as a control, and compared with all other groups ([a]Indomethacin, [b]L-NA, [c]CAPE).

Effect of fractions and compounds on HDF cell migration

To assess the capabilities of test sample on HDF cells migration, scratch wound assay was used. Principle of it is closely related to the proliferation phase of wound healing. An advantage of this method is it illustrates the potential of compounds on cells migration in a simulation of injury wound.[17] Migration of HDF cells was observed after the cells were challenged with ethanol extract and its fraction. Regarding the proliferative effects, the respondents of treated cells were like to the untreated control cells excepted ethyl acetate treated group. In this study, the ethanol extract, hexane- and chloroform fractions at concentration of 0.78, 0.78 and 1.56 µg/ml significantly stimulated cell migration at early of 24 h with corresponding wound closure of 33.6, 33.7 and 31.7%, respectively, as compared to untreated cells (21.7%). The cells were also stimulated to migrate and completely closed the wound on day 3. Whereas, the wound closure of ethyl acetate- and water fractions treated group were at 95.7 and 85.7%, respectively (Table 4, Figure 2). Table 5 shows the percentages of spreading of HDF cells into the scratched zone after being cultured with KG1-KG10. The migration of HDF cells was significantly stimulated by KG1 (59.2%), KG3 (58.8%), KG5 (64.7%), KG7 (57.6%) and KG9 (58.0%) on day 2 compared with untreated control cells (22.3%). Figure 3 shows the proliferation and migration of fibroblast cells after scratch and culture with KG1-10. The stimulations of KG1 and KG9 on fibroblasts were both proliferation and migration which were visualized by increasing number and continuous forward movement of cells into the artificial wound area on day 1 to day 3. In addition, on day 3, the treatment of KG1 (0.78 µM, 93.3%) and KG9 (1.56 µM, 90.1%) significantly increased cell migration rate higher than those of the untreated control cells (41.2%), allantoin (75.5%) and *Aloe vera* gel (70.1%) treated group. *Aloe vera* gel and allantoin were employed as the positive controls in this study. They have been used in cosmetic and several pharmaceutical purposes including wound healing preparation. *Aloe vera* promotes glucomannan to stimulate fibroblast growth factor and cell activity which resulting in cell proliferation, migration and also increase collagen production and secretion.[18,19]Allantoin regulates inflammatory response, fibroblast proliferation, collagen production and extracellular matrix synthesis.[20]

Effect of fractions and compounds on soluble collagen production by HDF cells

Dermal fibroblasts surrounding the wound are also known to play an important role in wound healing from the late phase of inflammation to epithelialize the wound. Moreover, fibroblasts secrete different growth factors particularly basic fibroblast growth factor (b-FGF), transforming growth factors-β1, together with cytokines such as IL-1, IL-6, IL-11 and TNF-α.[21,22] It involves in the synthesis of glycosaminoglycans and proteoglycans, the major components of extracellular matrix which simplify the granulation tissue formation.[23] Newly synthesize and deposit of collagen is also mainly by this cell. Meanwhile, in turn, the fragment released from degraded collagen enhances fibroblast and endothelial cells to angiogenesis and re-epithelialization. Collagen is essential protein to repair the deformity and recover the ordinary building block and function. In the early stage, collagen Type III is firstly synthesized, in the later an abundant of skin collagen Type I is by then. The presence of collagen rich element in wound, especially in this remodeling phase, helps to improve skin elasticity, rearrange the granulation tissue, and tensile strength.[2,3,24] Hence, an advantage of extract and isolated compounds on collagen type I synthesis from fibroblast was evaluated using *in vitro* model. Treatment with various concentrations of fractions and KG1-10 resulted to significantly increased type-I collagen production from HDF cells except KG3 and KG8 (Table 6). The efficient concentrations of samples that produce highest cell proliferation were selected to study on this experiment. Total amount of secreted type-I collagen by HDF cells after treating was between 14.2 and 44.0 µg/ml. The ethanol extract (0.78 µg/ml) and hexane fraction (0.78 µg/ml) and KG2 (6.25 µM) significantly increased the collagen content (44.0, 35.3 and 38.7 µg/ml, respectively) compared with the control group, allantoin and *Aloe vera* gel (21.7, 23.0 and 29.4 µg/ml, respectively). In addition, collagen content in HDF cells treated with KG1, KG4-7 and KG9-10 were significantly higher than allantoin though it comparable to that of the *Aloe vera* gel. These results implied that hexane fraction and its isolated compounds especially KG2 are efficient to induce type I collagen synthesis which accelerate wound repair.

Table 2: HDF cell proliferation of ethanol extract and its fractions from *Kaempferia galanga*

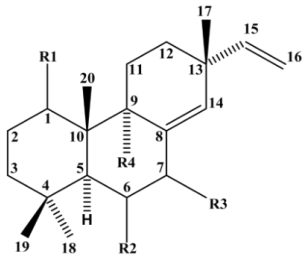
Samples	% Cell proliferation at various concentrations (µg/ml)						
	0	0.78	1.56	3.12	6.25	12.5	25
Control	100.0±1.1	-	-	-	-	-	-
Ethanol extract		83.0±2.8	78.9±3.4	75.8±4.2	74.2±3.6	77.2±5.0	70.2±5.0
Hexane fraction	-	95.5±2.8	94.1±1.9	92.8±1.8	85.5±8.9	80.5±8.9	71.2±6.0
Chloroform fraction	-	101.7±0.7	93.4±2.6	87.7±4.4	83.2±4.1	75.2±4.0	99.1±2.0
Ethyl acetate fraction	-	108.3±4.0*	106.5±4.2*	101.4±7.6	100.4±7.6	95.9±9.1	89.2±6.8
Water fraction	-	86.4±4.9	86.3±5.7	82.6±7.1	82.3±6.4	83.0±6.6	82.0±6.6

Each value represents mean±S.E.M. of three determinations.
*Statistical significant difference between control and various concentrations of sample at $p< 0.05$.

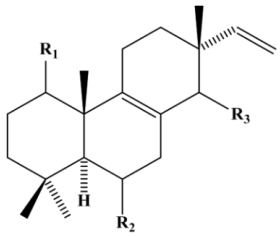
Table 3: HDF cell proliferation of compounds KG1-10 from *Kaempferia galanga*

Samples	**% Cell proliferation at various concentrations (µM)**						
	0	**0.78**	**1.56**	**3.12**	**6.25**	**12.5**	**25**
Control	100.0±0.0						
KG 1	-	99.0±4.0	98.0±2.6	93.3±2.9	95.4±2.8	89.9±2.6	88.1±0.3
KG 2	-	92.1±3.7	91.8±3.3	94.1±1.1	96.4±3.1	94.9±2.1	91.5±3.5
KG 3	-	95.8±1.6	102.3±0.6	98.5±0.3	98.5±0.5	97.3±1.9	90.8±3.1
KG 4	-	88.3±2.6	93.8±1.5	93.5±2.2	91.7±0.9	94.4±2.3	83.9±2.4
KG 5	-	104.8±4.5	106.2±4.5*	102.2±4.3	100.0±4.2	100.0±4.2	91.8±1.7
KG 6	-	105.1±4.5	101.4±4.3	104.2±4.4	112.7±4.8*	116.5±5.0*	97.3±1.1
KG 7	-	104.6±5.9	109.6±4.3*	106.±4.6	102.4±3.7	102.8±5.1	98.5±2.1
KG 8	-	106.3±4.5	107.2±5.8*	109.1±3.9*	114.0±4.2*	119.7±5.1*	116.8±2.2*
KG 9	-	101.1±4.7	101.9±4.9	100.1±4.1	97.3±3.9	94.9±5.4	85.1±4.4
KG 10	-	98.9±5.5	100.0±6.7	100.1±6.8	102.2±5.8	106.1±5.0	99.0±3.1
Allantoin	-	102.4±4.6	99.8±3.1	99.1±1.7	96.7±1.6	94.5±1.9	97.1±2.1
Aloe vera gel (µg/ml)	-	90.9±2.9	95.6±3.9	94.4±4.7	96.5±5.0	98.5±2.1	97.3±2.2

Each value represents mean±S.E.M. of three determinations.
*Statistical significant difference between control and various concentrations of sample at $p< 0.05$.



	R_1	R_2	R_3	R_4
KG1	H	H	H	H
KG2	H	β-$OCOCH_3$	H	OH
KG3	H	H	α-OH	OH
KG4	α-OH	H	H	OH
KG5	=O	β-$OCOCH_3$	H	OH
KG6	α-OH	β-$OCOCH_3$	H	OH



KG7	α-OH	H	α-OCH_3
KG8	H	β-OH	α-OH
KG9	α-OH	H	α-OH
KG10	H	β-OH	β-OH

Figure 1: Ten isopimarane diterpenoids isolated from *Kaempferia galanga*

Table 4: HDF cell migration of ethanol extract and its fractions from *Kaempferia galanga*

Samples	Dose (µg/ml)	Length between the scratch (µm)				%Migration rate of cells		
		Day 0	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Control	-	1142.3±40.5	894.8±42.6	576.6±61.2	348.2±4.0	21.7±1.9	49.8±3.6	63.1±1.6
Ethanol extract	0.78	925.1±31.4	616.5±61.8	217.6±47.6	0.0±0.0	33.6±5.0*	77.5±4.4*	100.0±0.0*
Hexane fraction	0.78	1036.5±64.7	357.4±36.7	95.6±7.3	0.0±0.0	33.7±0.6*	65.2±5.5*	100.0±0.0*
Chloroform fraction	1.56	1062.5±54.4	698.7±11.1	342.9±34.1	0.0±0.0	31.7±2.6*	68.1±3.9*	100.0±0.0*
Ethyl acetate fraction	0.78	1097.1±16.5	828.6±16.7	534.7±5.5	46.9±31.1	24.5±0.8	51.3±0.2	95.7±2.8*
Water fraction	0.78	1007.7±10.9	707.9±24.5	354.2±15.9	111.0±6.9	29.7±3.1	64.8±1.9*	89.7±0.8*

Each value represents mean±S.E.M. of three determinations.
*Statistical significant difference between control and various concentrations of sample at $p < 0.05$.

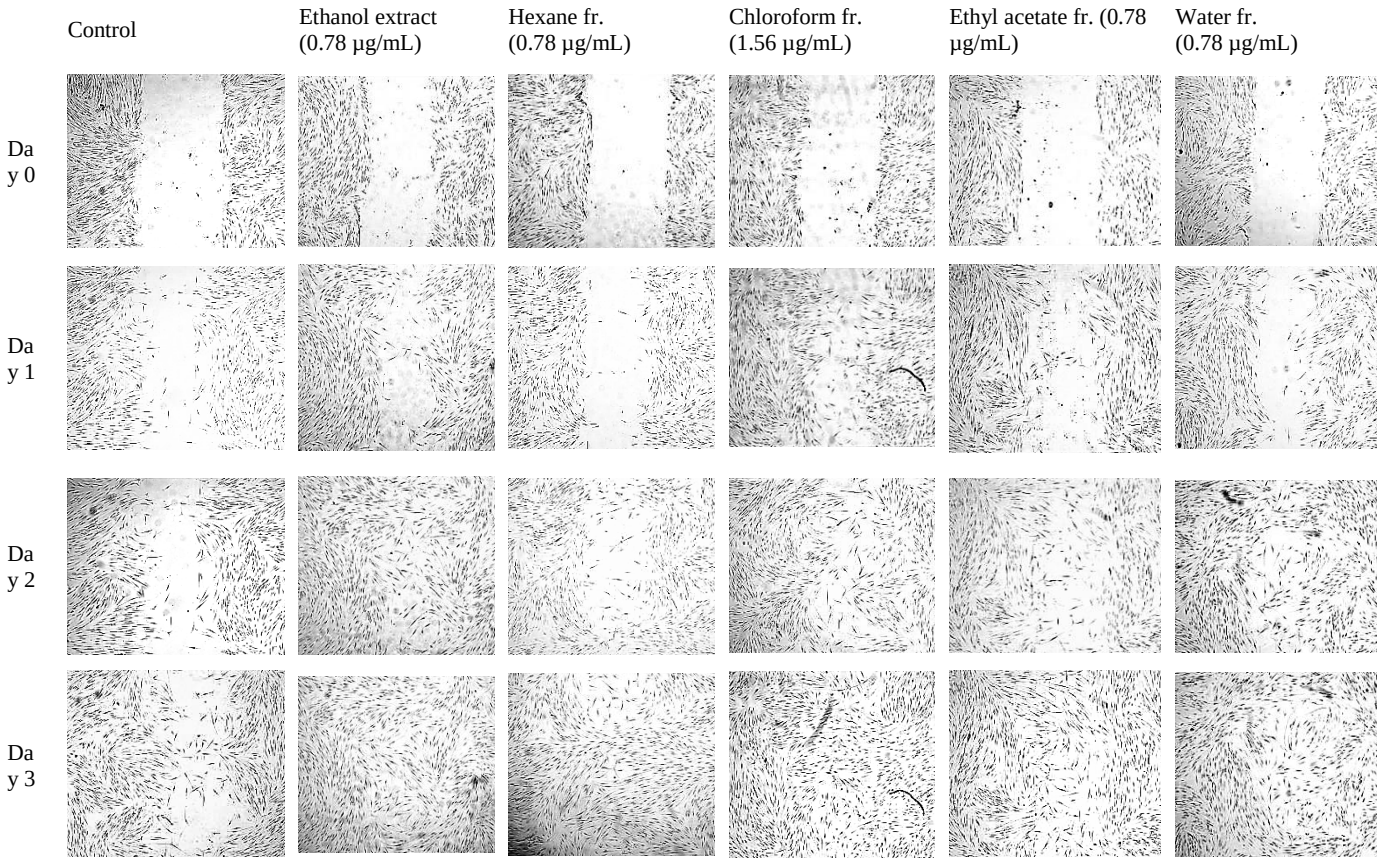
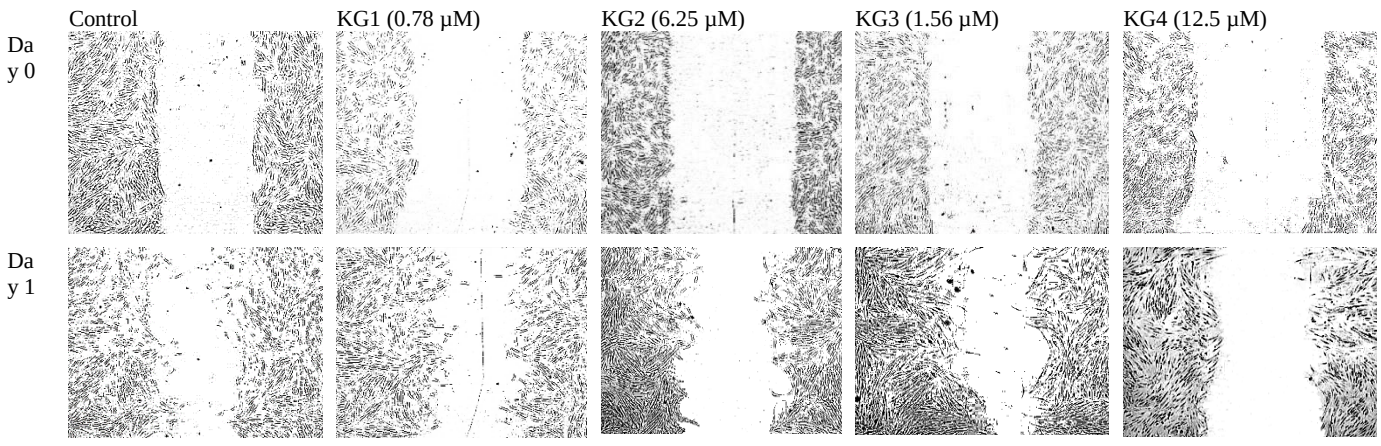


Figure 2: Effect on HDF cell migration of ethanol extract and its fractions from *Kaempferia galanga*



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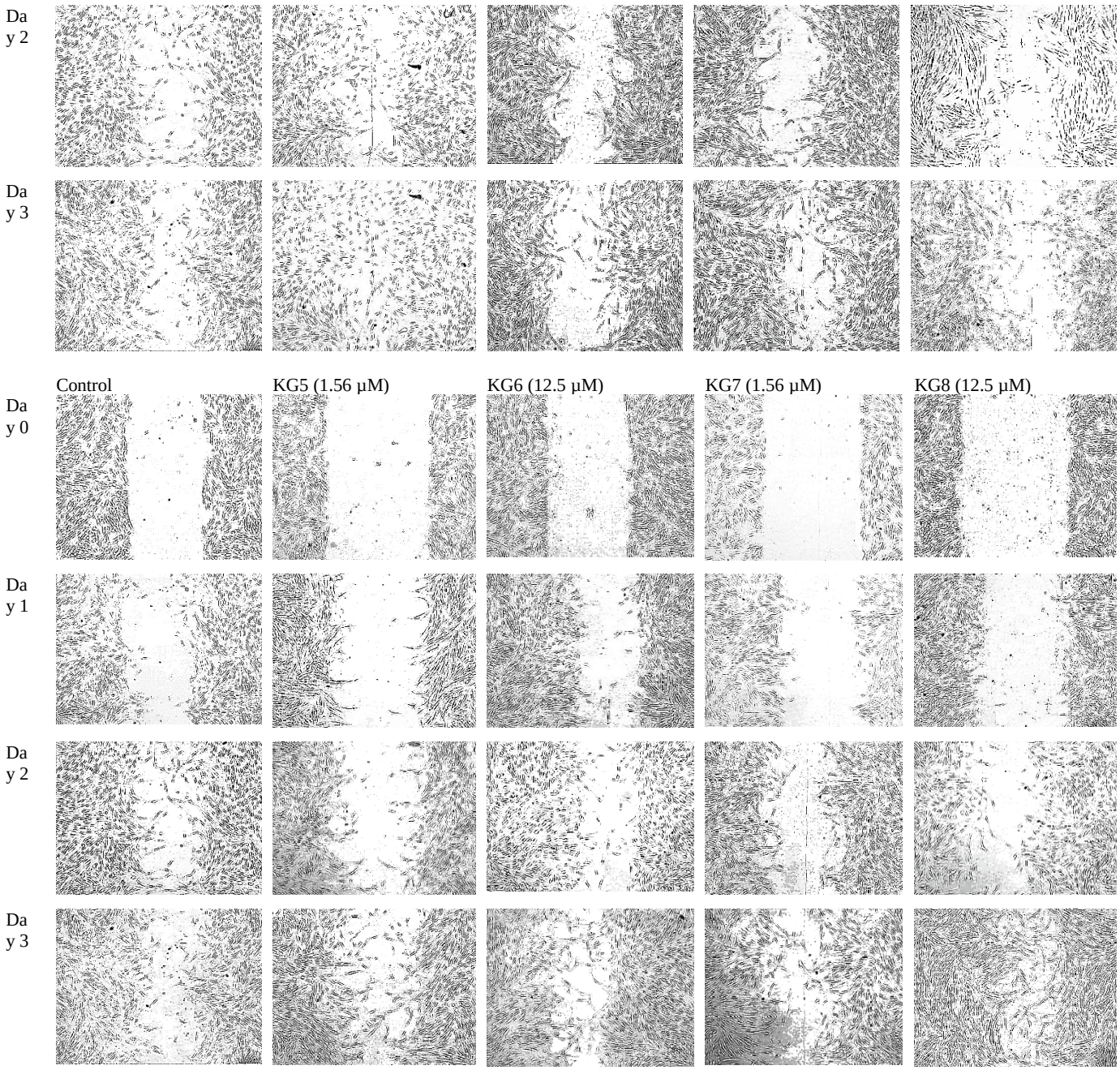
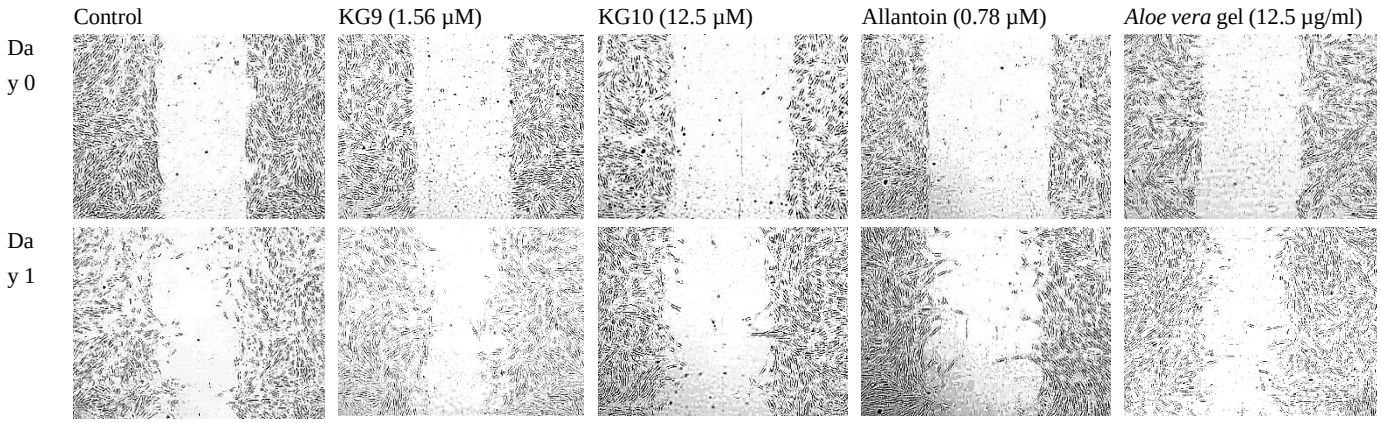


Figure 3: Effect of compounds KG1-10 from *Kaempferia galangal* on HDF cell migration



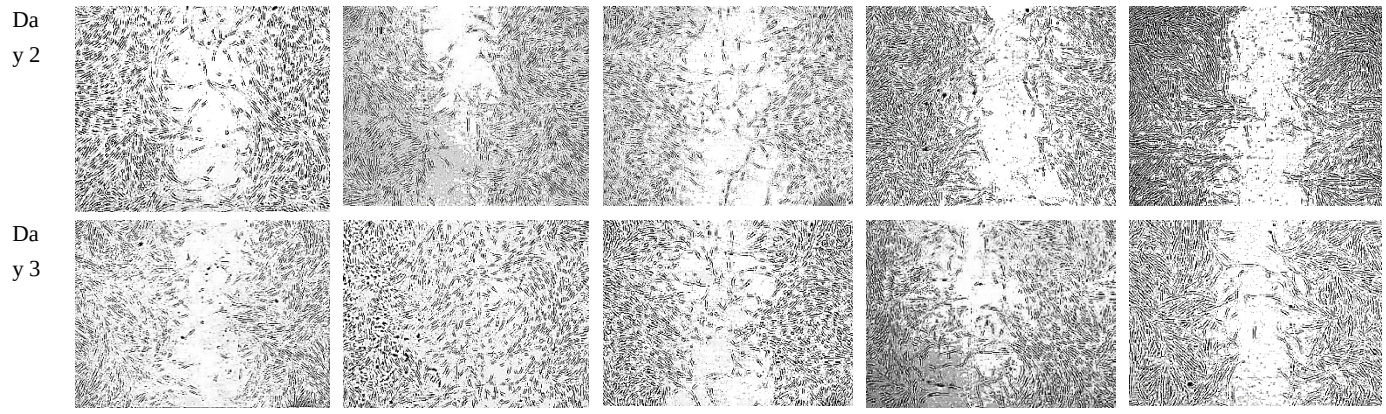


Figure 3 Cont'd: Effect of compounds KG1-10 from *Kaempferia galangal* on HDF cell migration

Effect of fractions and compounds on H_2O_2-induced oxidative stress in HDF cells

Impairment of wound healing leads to chronic wound and malfunction of organ. During inflammation, the production of reactive oxygen species (ROS) creates the oxidative damage to pathogen. However, the excessive accumulation of these reactive oxygen species including superoxide anion ($O^{\bullet -2}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), singlet oxygen (O_2) and reactive nitrogen species (RNS); nitric oxide (NO) and peroxynitrite ($ONOO^-$) over antioxidant capacity of cells leads to oxidative stress. The oxidative stress mediated by these molecules inhibit cell migration and proliferation, damage surrounding tissue and persistent of inflammation.[5,25,26] Accordingly, antioxidant capacity of cells is one of the important factors for success in the treatment of wound. In *in vitro*, an antioxidant study by measure fibroblast cell viability after disrupts it with strong oxidizer such as H_2O_2 in the presence or absence of test sample is regarded to correlate in the *in vivo* condition.[5] Therefore, H_2O_2-induced oxidative stress was applied in the antioxidant study of KG1-10. Base on amount of cell viability of KG1-10 treated group was similar to that of the control group (Table 3). In MTT assay, the HDF cells treated with samples not revealed adverse effect. Excess exogenous H_2O_2-induced oxidative stress decreased the cell viability of HDF cells. After incubated HDF cells with 0.9 mM H_2O_2 for 24 h, the cells viability was decreased to 53.1% when compared with that of the untreated control group. Conversely, the viability of cells co-treated with various concentrations of fractions (3.12-25 µg/ml) and compounds (3.12-25 µM) were restored as shown in Table 7. Ethanol extract significantly increased cell viability (66.7-77.2%) compared with the exposure cells to H_2O_2 alone. This effect also found in ethyl acetate and water fractions treated group that increased cell viability (64.5-83.7%) by countering the H_2O_2-induced oxidative stress. Co-cultured with KG2-10 at concentrations of 3.12-25 µM significantly increased cell viability (69.8-92.1%) compared with those of H_2O_2 treated alone. These effects were comparable to the positive control, allantoin (70.1-81.0%). Among these, KG7 exhibited the highest cell viability (75.4-92.1%) than the others which were implied the best anti-oxidative stress activity. This result indicated that the presence of diterpene compounds may enhance rapid wound healing by decreasing oxidative stress produced by intracellular free radicals. Superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase are enzymes that well counteract ROS into the redox balance. In the cells, H_2O_2 is produced by NOX/SOD signaling and converted by catalase and glutathione peroxidase to water and oxygen. These enzymes are therefore important to protect cells against oxidative stress.[25] The cytoprotective effect of isopimarane diterpene compounds, especially KG7, presented in this study may due to the influence on up-regulation of antioxidants enzymes participated in the neutralization. The productive effect of KG7 could be used as antioxidant in wound healing.

GC analysis of the hexane fraction from K. galanga rhizomes

KG1-10, isopimarane diterpenes isolated from the hexane fraction of *K. galanga* were subjected to GC analysis to obtain standard chromatograms. GC chromatogram of the hexane fraction and standard KG1-10 are shown in Figures 4 and 5. The retention time (min) and percentage of peak area of KG1-10 present in the hexane fraction are shown in Table 8. The chromatogram demonstrated that the hexane fraction is a rich source of diterpenes KG1-10 (49.73%), which are highly abundant in *K. galanga* rhizome. Moreover, this fraction also presented five peaks ascribed to unknown compounds at retention time of 9.956, 15.051, 15.402, 16.375 and 17.213 min with peak area 1.51, 6.75, 2.81, 12.45 and 8.02%, respectively. It would be interesting to elucidate the structure of these compounds and determine whether they possess any wound healing activity, which could make them candidates for further investigations. Based on the result, it seems that GC analysis can be used for standardization of the *K. galanga* extract. In addition, KG1, KG6 and KG8 could be used as biomarkers for wound healing study.

From these results, the hexane fraction of *K. galanga* exhibited good wound healing properties due to its constituents, compounds KG1-10. KG6 and KG8 exhibited the most potent anti-inflammatory activity. KG1, KG6, KG8 and KG9 appeared to promote human dermal fibroblast proliferation and migration activities at low doses in the second phase of cellular proliferation, while KG2 significantly increased the collagen content. KG7 exhibited the highest anti-oxidative stress activity by increasing cell viability. Furthermore, the GC chromatogram of the hexane fraction can be used for standardization of the *K. galanga* extract; and these compounds could be used as the biomarkers for wound healing study, especially KG6 and KG8. Most of *Kaempferia* plant extracts and isolated compounds have been demonstrated to stimulate cell proliferation, regeneration and rehabilitation in damaged tissues. Some compounds have been reported their benefit in cancer treatment by inhibit cell proliferation and neoplastic progression. Conversely, some of them have been reported weak anticancer activities against various cancer cells.[9-11] However, several compounds among them exhibited a potent anti-inflammatory[27,28] and antimicrobial activities.[11] Moreover, previous reports have suggested that the isopimarane terpenoids stimulated the growth of fibroblasts. [2,14] The results from this study also support these scientific findings

Table 5: HDF cell migration of compounds KG1-10 from *Kaempferia galanga*

Samples	Dose (µM)	Length between the scratch (µm)				%Migration rate of cells		
		Day 0	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Control	-	1055.1±25.6	874.7±21.4	818.7±11.5	620.5±15.0	17.1±0.6	22.3±1.9	41.2±0.3
KG 1	0.78	860.6±105.7	594.9±113.0	360.7±98.2	58.5±37.2	31.1±11.0	59.2±9.1 [a]	93.3±4.6[a,b,c]
KG 2	6.25	1001.2±89.8	737.0±73.5	206.6±80.9	206.6±80.9	26.5±1.3	51.7±10.9	80.2±6.4 [a]
KG 3	1.56	858.7±31.7	565.2±24.6	346.5±95.5	98.6±22.0	33.9±4.6	58.8±12.8 [a]	88.3±3.0 [a]
KG 4	12.5	1095.0±90.1	805.4±106.1	663.9±130.8	323.0±75.5	27.1±4.1	40.6±7.6	70.9±5.5 [a]
KG 5	1.56	1011.0±103.2	689.0±127.8	365.6±81.9	180.6±81.3	33.0±5.7	64.7±4.3 [a]	83.3±5.9 [a]
KG 6	12.5	872.0±27.0	626.4±15.9	420.3±20.1	101.5±21.9	28.1±0.4	51.6±3.8	88.5±2.2 [a]
KG 7	1.56	972.1±57.2	727.8±14.8	418.1±84.9	205.4±99.6	26.0±8.5	57.6±6.4 [a]	79.8±8.7 [a]
KG 8	12.5	862.6±47.0	573.3±128.6	440.3±128.5	142.2±74.4	34.8±12.0	50.3±13.1	84.4±8.1 [a]
KG 9	1.56	852.6±35.1	536.7±81.8	358.1±18.6	86.1±28.4	37.5±7.3	58.0±0.8 [a]	90.1±2.9[a,b,c]
KG 10	12.5	975.7±69.7	705.3±104.3	541.7±107.3	333.1±97.4	28.5±6.5	45.3±7.6	66.7±8.0 [a]
Allantoin	0.78	1006.7±16.9	658.3±57.5	578.3±32.8	246.7±20.5	34.7±5.4	42.5±4.0	75.5±1.9 [a]
Aloe vera gel (µg/ml)	12.5	1032.2±137.3	744.4±100.8	583.0±103.0	310.6±55.7	27.9±2.8	43.6±7.8	70.1±2.1 [a]

Each value represents mean±S.E.M. of three determinations.
Statistical significant difference between control and various concentrations of sample at $p< 0.05$ ([a]control, [b]Allantoin, [c]*Aloevera* gel).

Table 6: Type-1 collagen production in HDF cells when treated with extract, fractions and compounds from *Kaempferia galanga*

Sample	Dose (µM)	Type-I collagen (µg/ml)	Sample	Dose (µM)	Type-I collagen (µg/ml)
Control	-	21.7±0.1	KG4	12.5	29.2±0.6[a,b]
Ethanol extract	0.78(µg/ml)	44.0±0.3[a,b,c]	KG5	1.56	25.7±0.1[a,b]
Hexane fraction	0.78 (µg/ml)	35.3±0.4[a,b,c]	KG6	12.5	28.1±0.2[a,b]
Chloroform fraction	1.56 (µg/ml)	30.2±0.1[a,b]	KG7	1.56	29.0±0.0[a,b]
Ethyl acetate fraction	0.78 (µg/ml)	28.5±0.3[a,b]	KG8	12.5	14.2±0.1
Water fraction	0.78 (µg/ml)	23.9±0.2[a]	KG9	1.56	26.0±0.5[a,b]
KG1	0.78	25.5±0.0[a,b]	KG10	12.5	28.3±0.6[a,b]
KG2	6.25	38.7±0.0[a,b,c]	Allantoin	0.78	23.0±0.4[a]
KG3	1.56	18.0±0.1	*Aloe vera* gel	12.5 (µg/ml)	29.4±0.8[a,b]

Each value represents mean±S.E.M. of three determinations.
Statistical significant difference between IC_{50} of positive control and sample at $p< 0.05$. Dunnett t-tests treated one group as a control, and compared with all other groups ([a]Control ;[b]Allantoin, [c]*Aloevera*gel).

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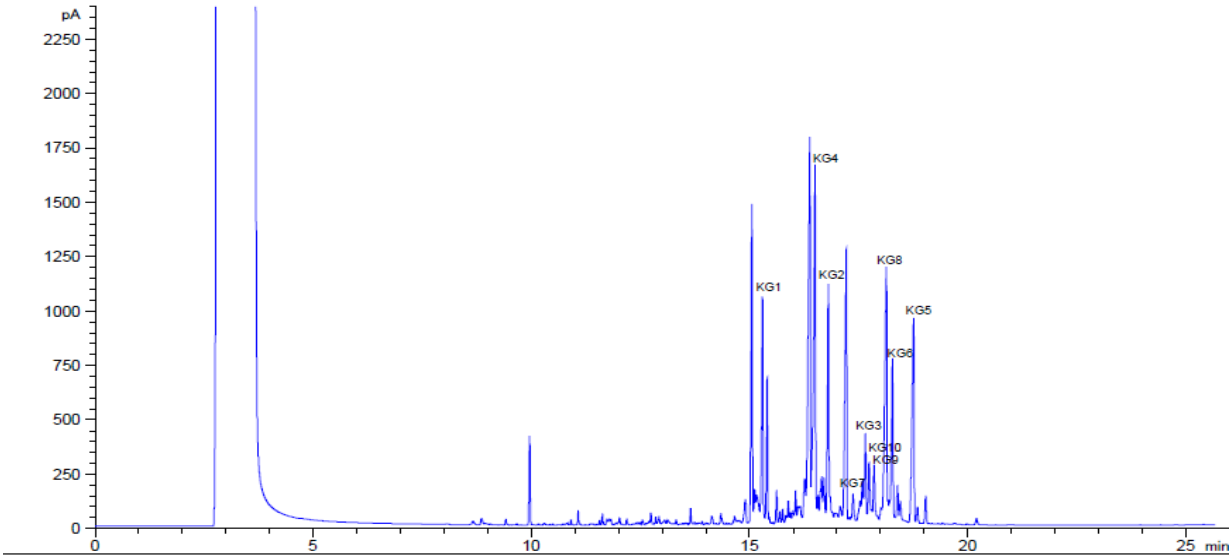
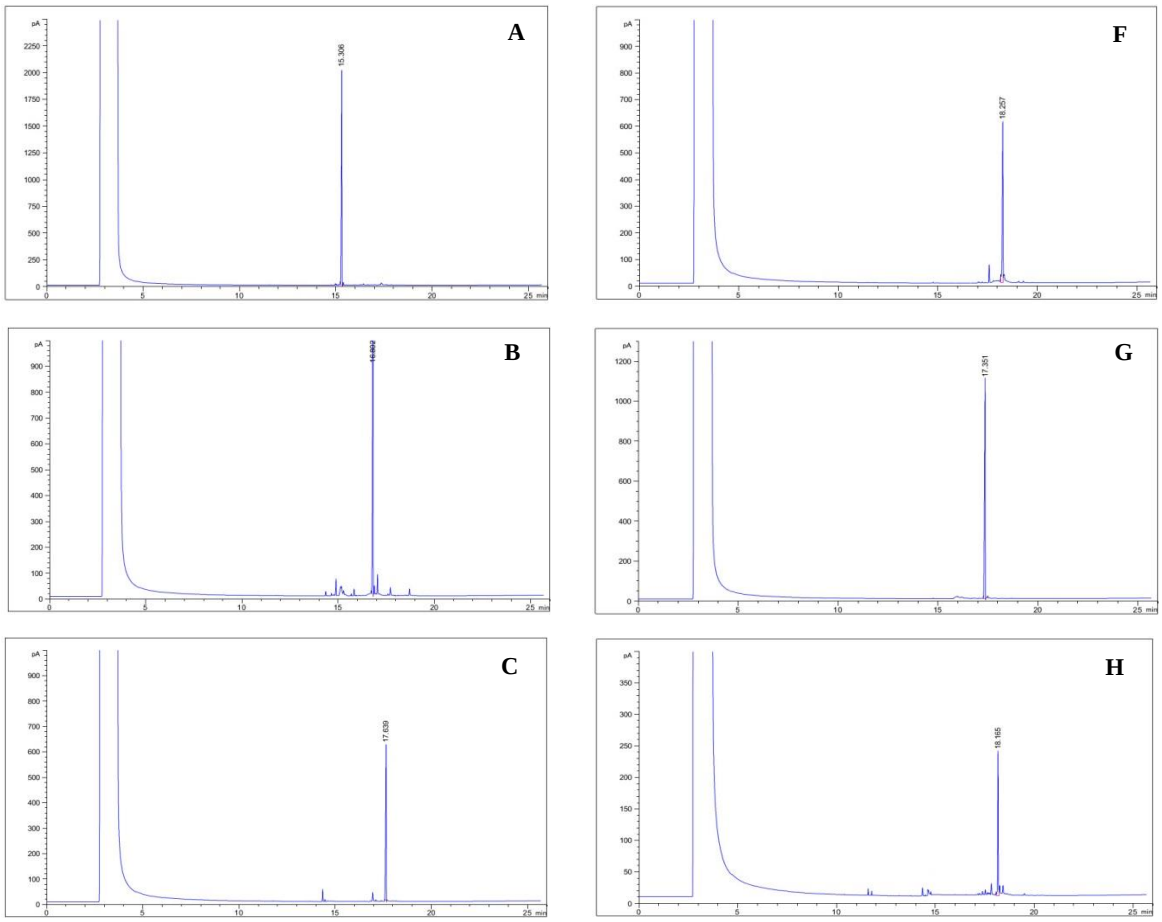


Figure 4: GC chromatogram of KG1-10 isolated from the hexane fraction of *Kaempferia galanga* rhizomes



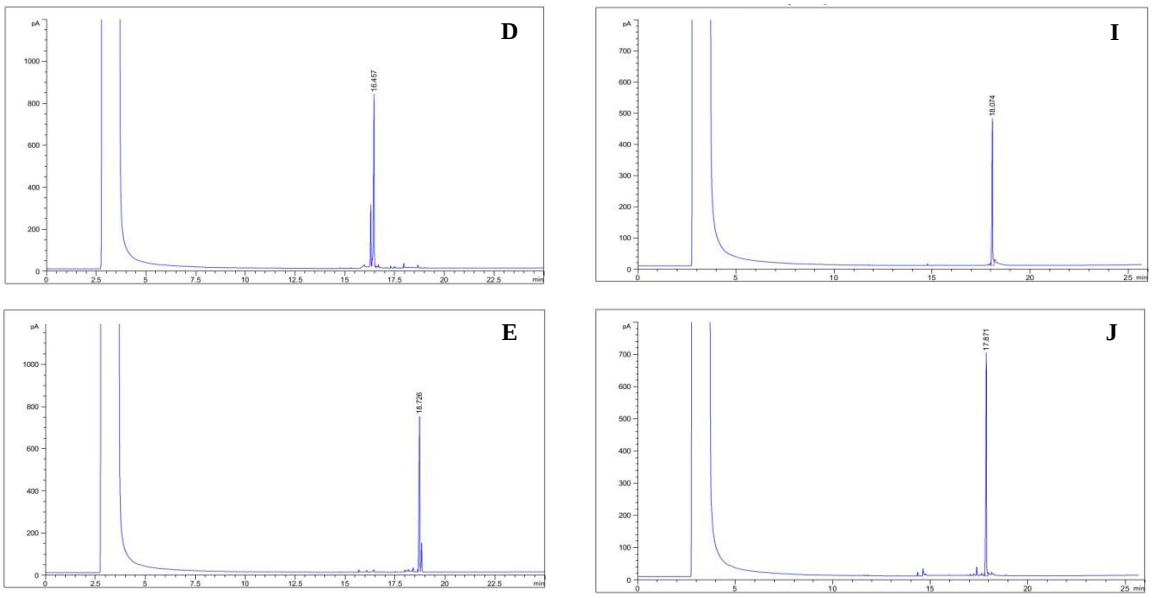


Figure 5: GC chromatogram of standard KG1-10 isolated from the hexane fraction of *Kaempferia galanga* rhizomes. A, B, C, D, E, F, G, H, I and J represent the GC chromatograms of KG1, KG2, KG3, KG4, KG5, KG6, KG7, KG8, KG9 and KG10, respectively.

Table 7: Protective effect of isolated compounds from *K. galanga* on H_2O_2-induced oxidative stress in HDF cells

Samples	% Cell proliferation at various concentrations (µM)				
	0	**3.12**	**6.25**	**12.5**	**25**
Control	100.0±0.0				
H_2O_2	53.1±1.9				
Ethanol extract		77.2±6.4*	77.5±3.6*	66.7±2.4*	61.4±3.5
Hexane fraction		67.8±7.0	54.1±6.5	50.6±6.0	43.3±4.2
Chloroform fraction		65.3±7.4	63.6±6.5	55.8±5.1	49.6±3.5
Ethyl acetate fraction		82.0±3.0*	80.9±3.8*	78.1±2.4*	70.6±0.9*
Water fraction		64.5±2.1*	65.2±3.2*	75.2±0.9*	83.7±2.6*
KG1		64.9±5.4	69.3±9.1	69.5±7.2	62.8±3.8
KG2		73.2±5.7*	74.3±4.9*	76.3±6.4*	65.6±6.0
KG3		66.1±5.8	72.8±7.8	76.8±7.8*	70.1±2.2
KG4		75.9±5.9*	77.3±6.5*	73.6±6.4*	63.9±6.0
KG5		72.8±5.1*	83.7±2.0*	72.6±4.1*	75.6±2.3*
KG6		73.7±7.1*	70.9±5.4*	73.9±3.1*	68.4±1.2
KG7		77.9±7.4*	92.1±7.8*	80.7±2.8*	75.4±5.2*
KG8		80.9±1.2*	75.2±3.4*	69.8±2.5*	61.8±7.1
KG9		75.7±5.2*	76.6±4.3*	76.0±0.7*	74.3±0.2*
KG10		80.6±5.1*	84.0±7.4*	89.6±8.1*	83.1±7.5*
Allantoin		75.2±1.6*	81.0±0.4*	75.4±2.7*	70.1±5.3*

Each value represents mean±S.E.M. of three determinations.
*Statistical significant difference between H_2O_2 treated and various concentrations of sample at $p< 0.05$. Dunnett's test treated one group as a control, and compared with all other groups.

Table 8: Retention time, peak area% and compounds isolated from *K. galanga* rhizomes using GC analysis

Retention time (min)	Peak area %	Sample name
15.290	5.05	(-)-sandaracopimaradiene (KG1)
16.494	11.53	sandaracopimaradiene-1α, 9α-diol (KG4)
16.803	6.52	6β-acetoxysandaracopimaradiene-9α-ol (KG2)
17.369	1.04	1α-hydroxy-14α-methoxyisopimara-8(9),15-diene (KG7)
17.652	2.47	sandaracopimaradiene-7α, 9α-diol (KG3)
17.851	1.97	6β,14β-dihydroxyisopimara-8(9),15-diene (KG10)
18.010	0.54	1α,14α-dihydroxyisopimara-8(9),15-diene (KG9)
18.128	8.73	6β,14α-dihydroxyisopimara-8(9),15-diene (KG8)
18.271	4.64	6β-acetoxysandaracopimaradiene-1α, 9α-diol (KG6)
18.756	7.24	6β-acetoxysandaracopimaradiene-9α-ol-1-one (KG5)
Total	49.73	

Conclusion

The present study provides scientific evidence that supported the traditional use of *K. galanga* for wound treatment. The ethanol extract of *K. galanga* and hexane fraction exhibited good wound healing properties due to its constituents, KG1-10. These compounds are responsible for wound healing properties through anti-inflammation, fibroblast proliferation and migration, collagen synthesis together with anti-oxidative stress activities. Moreover, the GC analysis of this active isopimarane diterpenes from *K. galanga* rhizomes can be applied for further standardization of the extract and its fractions as biomarkers for wound healing study. Therefore, *K. galanga* extract and its compounds may be useful as potential cosmetic and pharmaceutical materials. Future studies should focus on evaluating the suitability of these extracts and compounds in cosmetic and pharmaceutical formulations to enhance their value for wound healing and skin regeneration purposes.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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